

Supplemental Figure 1 (related to Figure 1)

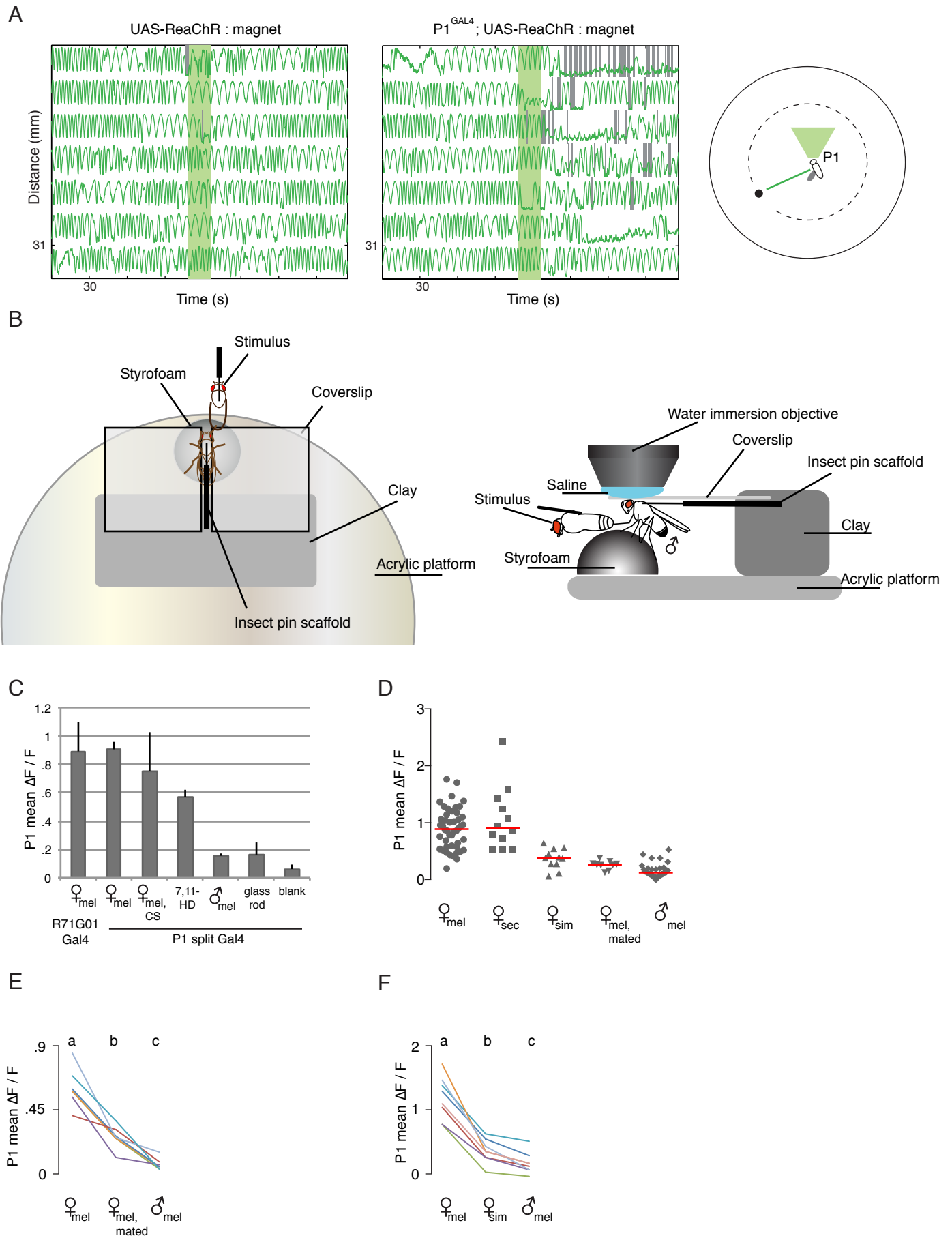
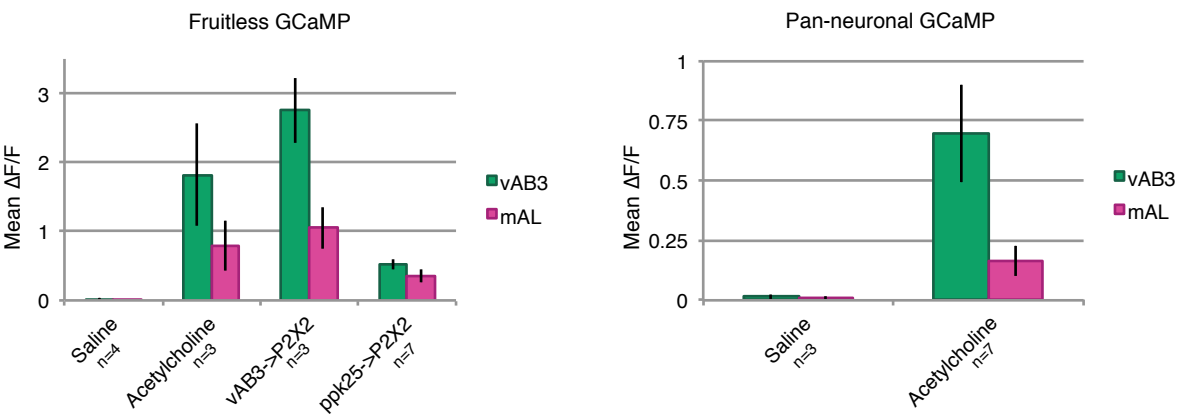
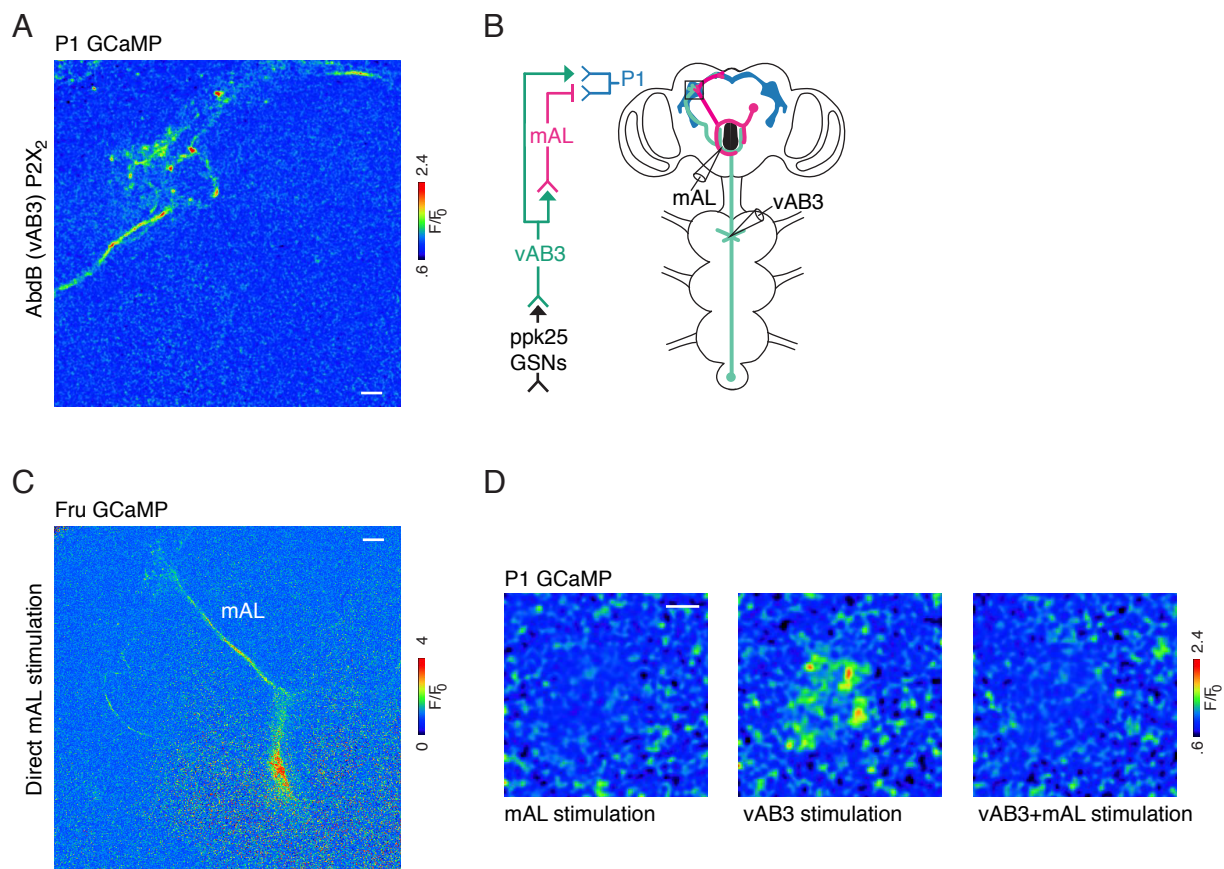


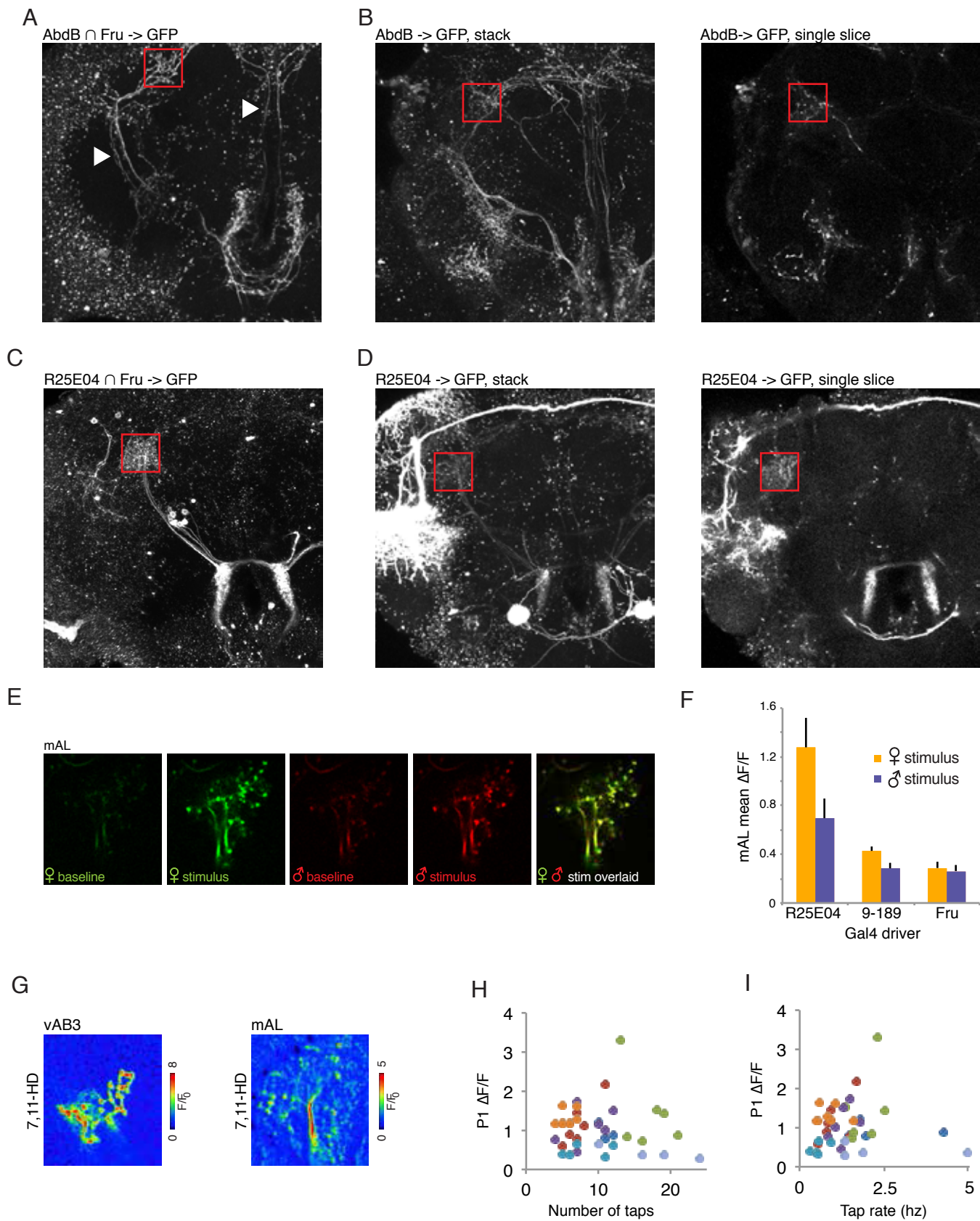
Figure S2 (related to Figure 2)



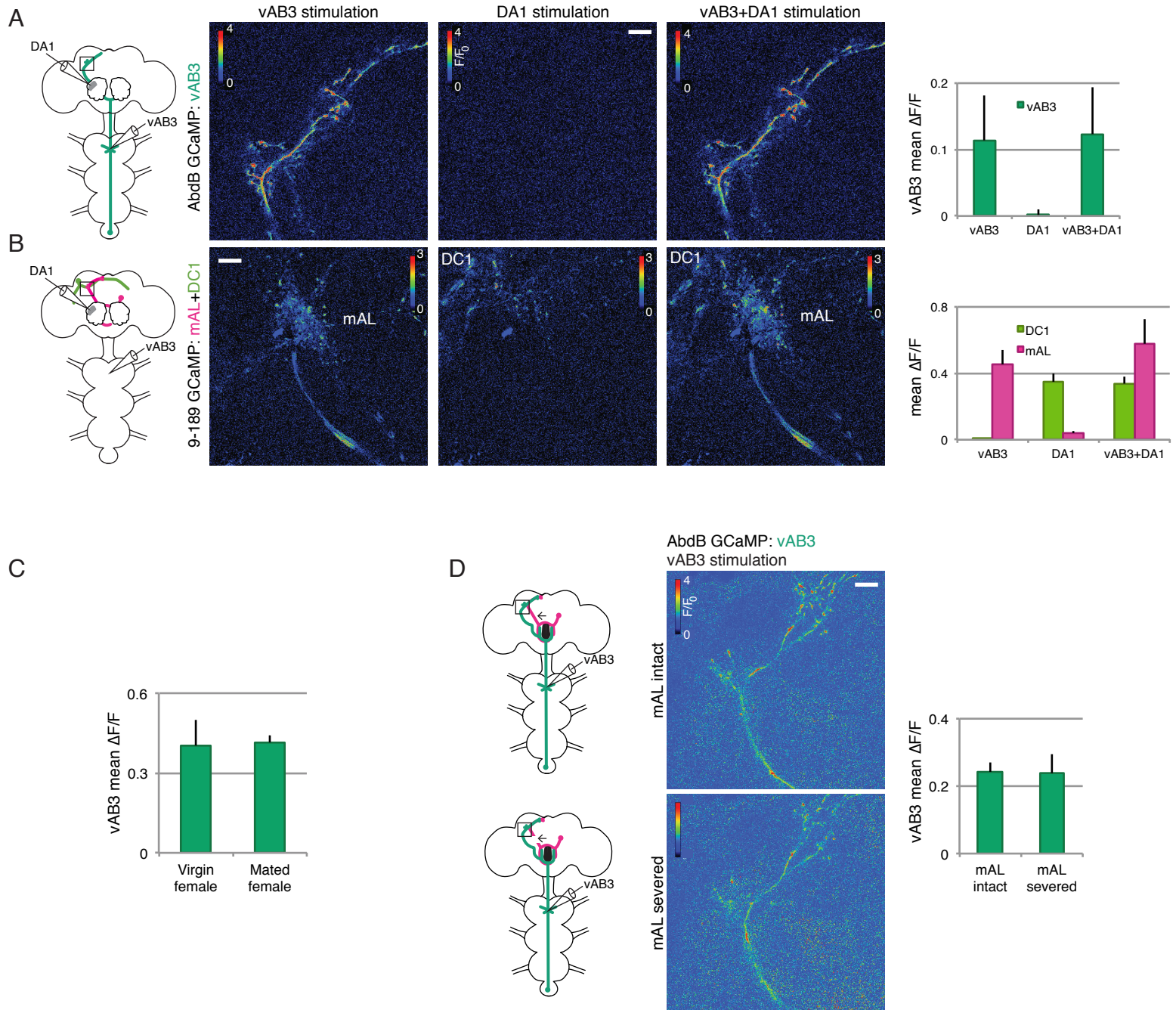
Supplemental Figure 3 (related to Figure 3)



Supplemental Figure 4 (related to Figure 4)



Supplemental Figure 5 (related to Figure 6)



Supplemental Figure Legends

Figure S1 (related to Figure 1)

- (A) Fly-magnet distance (green) and wing extension events (grey) for seven individual assays shown averaged in Figure 1C. Precise oscillation in distance traces occurs when the male stands still and the magnet rotates around him.
- (B) Schematic and images of *in vivo* imaging preparation, as described in Figure 1E and Extended Experimental Procedures.
- (C) Average P1 neuron GCaMP responses to indicated stimuli recorded in neurons labeled by *P1-Gal4* or, where indicated, *R71G01-Gal4* (n=2). “Blank” indicates P1 GCaMP time series during which no stimulus was presented. n=2 animals for Canton S female stimulus (CS) and glass rod, n=3 for 7,11-HD and blank, n=40 for *melanogaster* male and female (reproduced from Figure 1).
- (D) Individual animal mean P1 GCaMP responses to indicated stimuli, shown averaged in Figure 1J. Red line indicates median.
- (E-F) Individual animal paired mean P1 GCaMP responses to indicated stimuli recorded in neurons labeled by *P1-Gal4*. Responses to *D. simulans* females and mated *melanogaster* females always fell between male and virgin female *melanogaster* responses. Significance, ANOVA with Tukey’s correction.

Figure S2 (related to Figure 2)

Quantification of mean change in GCaMP fluorescence in mAL and vAB3 neurons for experiments in Figure 2E, F, H, and I. Responses are shown for saline control, acetylcholine stimulation of vAB3, or ATP stimulation of ppk25 or vAB3 expressing P2X₂. n for each experiment is displayed on the graph. Error bars depict SEM.

Figure S3 (related to Figure 3)

- (A) Multi-plane imaging of P1 neurons expressing GCaMP under control of *R71G01-LexA* in response to genetically restricted stimulation of *AbdB^{LDN}*-positive neurons expressing the P2X₂ channel and activated by local application of ATP in VNC.
- (B) Schematic describing direct activation of vAB3 and/or mAL neurons
- (C) Representative multi-plane imaging of Fru⁺ neurons expressing GCaMP in response to local application of acetylcholine on mAL dendrites in the SEZ. Within the Fruitless population, this activated only mAL and a single anterior neuron whose anatomy was not familiar to us. Representative of three experiments.
- (D) Functional imaging of P1 neurons in response to stimulation of mAL neurons (left), vAB3 neurons (middle) or both (right) by acetylcholine iontophoresis. P1 neurons express GCaMP under *R71G01-Gal4* and mAL dendrites were targeted using *Fru^{LexA}* to express Tomato. Representative of two experiments.

Figure S4 (related to Figure 4)

(A-D) Two photon stacks showing *AbdB^{LDN}* and *R25E04-Gal4* expression patterns in intersection with Fruitless (*Fru^{flp}*) (A, C) or driving GFP directly (B, D). Though the Gal4 lines label neurons in addition to vAB3 and mAL, the lateral protocerebral complex (boxed) could be unambiguously identified in *in vivo* imaging experiments. Arrowheads in (A) point to neurons labeled by UAS>stop>CD8-GFP; *AbdB^{LDN}/Fru^{flp}* that were not labeled using the tubulin>Gal80> intersectional strategy described in Figure 2C, I.

(E) Overlay of mAL neurons stimulated by male (green) and female (red) stimuli. Baseline and evoked fluorescence are shown. F/F0 was not used to minimize spatial artifacts created by motion. The anatomic similarity between mAL processes activated by male and female stimuli was consistent across all experiments.

(F) Comparison of mAL responses to male and female *D. melanogaster* stimuli recorded from neurons labeled with three different Gal4 drivers. Regardless of the driver, male and female stimuli both robustly activated mAL neurons. n=4-6 animals.

(G) Functional imaging of vAB3 or mAL neurons in response to stimulation with 7,11-HD deposited on a glass rod. Representative of 2 experiments each.

(H-I) P1 responses evoked by foreleg contact to a female stimulus are not correlated with the number of taps (H) or tap rate (I) within a bout. Color-coding indicates different bouts recorded in the same animal. 39 bouts scored from seven experiments. See also Figure 4G.

Figure S5 (related to Figure 6)

(A-B) Schematics, representative multi-plane functional imaging, and quantification of stimulation of vAB3 neurons (A, n=3), or mAL neurons and DC1 neurons (B, n=3) in response to stimulation of vAB3 neurons (left), DA1 projection neurons (middle) or both (right) by local acetylcholine iontophoresis. vAB3 neurons express GCaMP under *AbdB^{LDN}* and mAL and DC1 neurons express GCaMP under *9-189^{Gal4}*.

(C) vAB3 GCaMP responses evoked by foreleg contact with virgin or mated *melanogaster* female. n=5 animals.

(D) Schematic and representative multiplane functional imaging (n=3) of vAB3 neurons in response to vAB3 stimulation via acetylcholine iontophoresis before (top) or after (bottom) mAL severing. vAB3 neurons expressed GCaMP under control of *AbdB^{LDN}*, and *Fru^{LexA}* drove expression of Tomato to guide mAL severing.

Supplemental Movie

Example of *in vivo* imaging of P1 neurons. Side-view video imaging used to guide stimulation is aligned with P1 GCaMP two-photon times series. A female stimulus is presented and the male fly taps her with his foreleg. Heatmap displays F/F0 in P1 neurons expressing GCaMP under control of *P1-Gal4*.

Extended Experimental Procedures

Flies

Flies were housed under standard conditions on molasses/cornmeal agar at 23-25C under a 12 hr light: 12 hr dark cycle.

Strains and sources:

Drosophila melanogaster Canton S, Gr5a-Gal4 (#57592), UAS-ReaChR-Citrine (#53741), UAS-GCaMP6s (#42746, 42749), UAS-mCD8::GFP (#5130, #5137), LexAop-GCaMP6s (#53747), LexAop-FLP (#55820), UAS-myr::tdTomato (#32222), UAS>stop>mCD8::GFP (#30125), and R25E04-Gal4 (#49125) were obtained from the Bloomington Stock Center. The following were gifts, obtained as indicated: *Drosophila melanogaster* outcrossed (Tom Turner, UCSB); *Drosophila simulans* w501 and *Drosophila sechellia* (Richard Benton, University of Lausanne); LexAop-myr::tdTomato (Cesar Mendes Columbia University); PoxN-Gal4-13 (Markus Noll, University of Zurich); P1-Gal4 activation domain and P1-Gal4 DNA binding domain (Inagaki et al., 2014, David Anderson, Caltech); Fru^{LexA} (Mellert et al., 2010), R71G01-LexA, and R71G01-Gal4 (Jennet et al., 2012) (Bruce Baker, HHMI/Janelia Farm Research Campus); Fru^{Gal4} (Stockinger et al., 2005), Fru^{flp} (Yu et al., 2010), and Or67d^{Gal4} (Kurtovic et al., 2007) (Barry Dickson, HHMI/Janelia Farm Research Campus); AbdominalB^{LDN}-Gal4 (de Navas et al., 2006, Ernesto Sanchez-Herrero, Centro de Biología Molecular Severo Ochoa); ppk23-Gal4 (Thistle et al., 2012) and tub>Gal80> (Kristin Scott, UC Berkeley); ppk25^{Gal4} (Starostina et al., 2012, Claudio Pikielny, Dartmouth Medical School); Gr32a-Gal4 (John Carlson, Yale University); 9-189^{Gal4} (Ulrike Heberlein, HHMI/Janelia Farm Research Campus); nSyb-Gal4 (Julie Simpson, HHMI/Janelia Farm Research Campus). UAS-C3PA: (Ruta et al., 2010). LexAop-SPA-T2A-SPA, and UAS-P2X₂ were generated by standard cloning and injection (The Best Gene, Chino Hills, CA). P2X₂ sequence (gift of Gero Miesenbock, University of Oxford) was cloned into attB-modified pUAST and inserted into attP40 by phiC31 recombination, and SPA-T2A-SPA (Datta et al., 2008) was cloned into pLOT and inserted into the genome by P element transposition.

Detailed genotypes used in each figure; animals are male unless noted.

Figure 1A-B

Males: Canton S

Females: UAS-eGFP-kir2.1 or AbdB^{LDN} (control genotypes from Bussell et al., 2014)

Figure 1C

P1-Gal4 AD/UAS-ReaChR-citrine; P1-Gal4 DBD/TM2 or TM6B

Control: +/-UAS-ReaChR-citrine; +/-TM2 or TM6B

Figure 1D

UAS-C3PA/UAS-C3PA; Fru^{Gal4}/UAS-C3PA

Figure 1F-J

Male Subject: P1-Gal4 AD /UAS-GCaMP6s; P1-Gal4 DBD /UAS-GCaMP6s

Stimuli: *Drosophila melanogaster* outcrossed, *Drosophila simulans* w501, *Drosophila sechellia*

Figure 2A

PoxN-Gal4-13/ LexAop-myr::tdTomato; UAS-mCD8::GFP/ Fru^{LexA}

Gr5a-Gal4/ LexAop-myr::tdTomato; UAS-mCD8::GFP/ Fru^{LexA}

UAS-mCD8::GFP / LexAop-myr::tdTomato; ppk23-Gal4/ Fru^{LexA}

UAS-mCD8::GFP / LexAop-myr::tdTomato; ppk25^{Gal4} / Fru^{LexA}
UAS-mCD8::GFP / LexAop-myr::tdTomato; Gr32a-Gal4 / Fru^{LexA}

Figure 2B

UAS-C3PA/UAS-C3PA; Fru^{Gal4}/UAS-C3PA

Figure 2C

tub>Gal80>; LexAop-myr::tdTomato, LexAop-FLP / UAS-mCD8::GFP; AbdB^{LDN}-Gal4/Fru^{LexA}

Figure 2E

UAS-GCaMP6s/CyO; Fru^{Gal4}/TM2

Figure 2F

UAS-GCaMP6s/ UAS-GCaMP6s; nSyb-Gal4/ UAS-GCaMP6s

Figure 2G

UAS-C3PA/UAS-C3PA; Fru^{Gal4}/UAS-C3PA

Figure 2H

LexAop-GCaMP6s/UAS-P2X₂; Fru^{LexA}/ppk25^{Gal4}

Figure 2I

tub>Gal80>; LexAop-GCaMP6s, LexAop-FLP / UAS-P2X₂; AbdB^{LDN}-Gal4/Fru^{LexA}

Figure 3B

LexAop-SPA-T2A-SPA /UAS-myr::tdTomato; Fru^{LexA}/R71G01-Gal4

Figure 3C

P1-Gal4 AD/CyO; P1-Gal4 DBD, UAS-mCD8::GFP/TM6B

Figure 3D-F

UAS-GCaMP6s/LexAop-myr::tdTomato; R71G01-Gal4/ Fru^{LexA}

Figure 4A, C

Male Subject: UAS-GCaMP6s/ UAS-GCaMP6s; AbdB^{LDN}-Gal4/TM2

Stimuli: *Drosophila melanogaster* outcrossed

Figure 4B, D

Male Subject: UAS-GCaMP6s/ UAS-GCaMP6s; R25E04-Gal4/TM2

Stimuli: *Drosophila melanogaster* outcrossed

Figure 4F-G

Male Subject: P1-Gal4 AD/UAS-GCaMP6s; P1-Gal4 DBD/UAS-GCaMP6s

Female Stimulus: *Drosophila melanogaster* outcrossed

Figure 4H-I

UAS-GCaMP6s/LexAop-myr::tdTomato; R71G01-Gal4/ Fru^{LexA}

Figure 5

Male Subject: P1-Gal4 AD/UAS-GCaMP6s; P1-Gal4 DBD/UAS-GCaMP6s

Stimuli: *Drosophila melanogaster* outcrossed

Figure 6B, C

LexAop-SPA-T2A-SPA/UAS-myr::tdTomato; Fru^{LexA} /R71G01-Gal4

Figure 6D

LexAop-SPA-T2A-SPA/CyO; Fru^{LexA} /TM6B

Figure 6E, F

Male Subject: UAS-GCaMP6s/UAS-GCaMP6s; Fru^{Gal4}/TM6B

Stimuli: *Drosophila melanogaster* outcrossed

Figure 6G

Male Subjects:

P1-Gal4 AD/UAS-GCaMP6s; P1-Gal4 DBD/UAS-GCaMP6s

UAS-GCaMP6s/UAS-GCaMP6s; Or67d^{Gal4} (null allele), R71G01-Gal4/Or67d^{Gal4}, R71G01-Gal4

Female stimulus: *Drosophila melanogaster* outcrossed

Figure 6H

UAS-GCaMP6s/LexAop-myr::tdTomato; R71G01-Gal4/ Fru^{LexA}

Genotypes of flies related to Supplemental Figures

Animals are male unless noted.

Figure S1A

P1-Gal4 AD/UAS-ReaChR-citrine; P1-Gal4 DBD/TM2 or TM6B
+/UAS-ReaChR-citrine; +/-TM2 or TM6B

Figure S1C

Male subject:

P1-Gal4 AD/UAS-GCaMP6s; P1-Gal4 DBD/UAS-GCaMP6s
-except “R71G01-Gal4” is UAS-GCaMP6s / UAS-GCaMP6s; R71G01-Gal4/TM2

Stimuli are outcrossed *D. melanogaster*

-except where indicated “CS” is Canton S

Figure S1D-F

Male Subject: P1-Gal4 AD/UAS-GCaMP6s; P1-Gal4 DBD/UAS-GCaMP6s

Stimuli: *Drosophila melanogaster* outcrossed, *Drosophila simulans* w501, *Drosophila sechellia*

Figure S2

As shown in Figure 2

Figure S3A

R71G01-LexA/UAS-P2X₂; LexAop-GCaMP6s/AbdB^{LDN}-Gal4

Figure S3C

LexAop-GCaMP6s/CyO; Fru^{LexA} /TM2

Figure S3D

UAS-GCaMP6s /LexAop-myr::tdTomato; R71G01-Gal4 / Fru^{LexA}

Figure S4A

UAS>stop>mCD8::GFP/CyO; AbdB^{LDN}-Gal4/Fru^{flp}

Figure S4B

UAS-mCD8::GFP/CyO; AbdB^{LDN}-Gal4/TM6B

Figure S4C

UAS>stop>mCD8::GFP/+; R25E04-Gal4/Fru^{flp}

Figure S4D

UAS-mCD8::GFP/+; R25E04-Gal4/TM6B

Figure S4E

UAS-GCaMP6s / UAS-GCaMP6s; R25E04-Gal4 /TM2

Female stimulus: *Drosophila melanogaster* outcrossed

Figure S4F

Male subjects:

UAS-GCaMP6s / UAS-GCaMP6s; R25E04-Gal4/TM2

UAS-GCaMP6s/ UAS-GCaMP6s; 9-189^{Gal4}/TM2;

UAS-GCaMP6s /CyO; Fru^{Gal4} /TM6B

Stimuli: *Drosophila melanogaster* outcrossed

Figure S4G

UAS-GCaMP6s/ UAS-GCaMP6s; AbdB^{LDN}-Gal4/TM2

UAS-GCaMP6s / UAS-GCaMP6s; R25E04-Gal4/TM2

Figure S4H-I

P1-Gal4 AD/UAS-GCaMP6s; P1-Gal4 DBD/UAS-GCaMP6s

Female stimulus: *Drosophila melanogaster* outcrossed

Figure S5A

UAS-GCaMP6s / UAS-GCaMP6s; AbdB^{LDN}-Gal4/TM6B

Figure S5B

UAS-GCaMP6s /UAS-GCaMP6s; 9-189^{Gal4}/TM2

Figure S5C

Male Subject: UAS-GCaMP6s/ UAS-GCaMP6s; AbdB^{LDN}-Gal4/TM2

Female Stimulus: *D. melanogaster* outcrossed

Figure S5D

UAS-GCaMP6s /LexAop-myr::tdTomato; AbdB^{LDN}-Gal4/Fru^{LexA}

Courtship behavioral assays and analysis

Assays in Figure 1A are re-analyzed from Bussell et al., 2014. 4-7 day old males were tested with 1-2 day old virgin females in a modified 120 mm flybowl (Simon and Dickinson, 2010). Distance between flies was tracked using Ctrax (Branson et al., 2009); singing was manually scored.

Optogenetic stimulation

Male flies were separated from females after eclosion and aged for one day, then transferred to food containing 400uM all-trans-retinal and housed in the dark for 1 or 2 days before assays. Single male flies were loaded into a 38mm diameter, 6mm depth circular chamber with a small actuating Neodymium magnet (1.6×0.8 mm) rotating at a tangential velocity of 30 mm/sec. This design was inspired by (Agrawal et al., 2014): magnet actuation was controlled by another magnet beneath the chamber attached to a 57oz-in 1Nm NEMA Stepper Motor controlled by an Arduino Uno (Arduino) and EasyDriver Stepper Motor Driver (Sparkfun). The chamber was illuminated with a strip of blue LEDs (395 nm) to facilitate fly vision and video collection (Point Grey Firefly camera). A collimated LED (530 nm Precision LED Spotlight with Uniform Illumination –PLS-0530-030-S, Mightex Systems) was used to depolarize cells expressing ReaChR (Inagaki et al., 2014). In our setup, this LED was positioned to evenly illuminate the circular behavioral chamber at an intensity of 0.025mW/mm² at 530 nm. Each fly was introduced to the chamber and allowed to acclimate for 30 seconds before video recording began. Each fly was then recorded for the entirety of: 2 minutes prior to stimulation, 20 seconds of constant illumination, followed by another 2 minutes without stimulation. Distance between fly and the magnet for each assay was tracked using Ctrax (Branson et al., 2009); singing was manually scored.

Two-photon microscopy

All imaging experiments were performed on an Ultima two-photon laser scanning microscope (Bruker Nanosystems) equipped with galvanometers driving a Chameleon Ultra II Ti:Sapphire laser. Emitted fluorescence was detected with either photomultiplier-tube or GaAsP photodiode

(Hamamatsu) detectors. Images were acquired with an Olympus 60 \times , 0.9 numerical aperture objective at 512 pixels \times 512 pixels resolution.

Live imaging and data analysis

To prepare flies for *in vivo* imaging, CO₂-anesthetized 2-10 day old males were affixed to a pin with UV curable glue and given a Styrofoam ball to hold. After two hours recovery in a humidified chamber (and without further anesthesia), flies were mounted in a large acrylic disc with a small well to support the ball using modeling clay and UV glue as depicted in Figure 1E and Figure S1B. Glue was cured in short bursts to minimize exothermic damage to the preparation. Angled coverslips were supported by the clay and affixed to the fly's head and thorax using UV glue to create a vessel for saline. A small hole in the head was opened under perfusion saline (103mM NaCl, 3mM KCl, 5mM TES, 26mM NaHCO₃, 1mM NaH₂PO₄, 1.5mM CaCl₂, 4mM MgCl₂, 10mM Trehalose, 10mM glucose, osmolarity adjusted to 275 mmol/Kg) using sharp forceps. Surface tension typically prevented saline from spilling off the edges of the cover glass, however, if saline spilled onto the antenna or foreleg, the experiment was immediately terminated. Only animals that exhibited robust walking or grooming behavior following dissection were used for further experimentation. Animals were then placed beneath the objective in the two-photon microscope. 850nm LED lights were used to illuminate the chamber, and the fly was imaged from the side to guide and record stimulation using a Point Grey Firefly camera mounted with a 1x-at-94mm Infinistix lens fitted with a shortpass IR filter (850nm OD 4, Edmund Optics) to block 925nm two photon laser illumination.

Stimuli mounted on micromanipulators were presented in sets of 3-6 replicated touch bouts every 30 seconds. Virgin female *D. melanogaster* stimuli were usually presented first to assess sample integrity; preparations with no response to virgin female positive control were not analyzed further. In a subset of experiments, we presented the male stimulus initially to control for potential effects of stimulus order; we observed no differences in responses to male and female stimuli upon changing their presentation order.

The stimulus fly was presented for 2-12 seconds (median 5) allowing multiple touches (median 10 for those in which tapping events were quantified post hoc), then withdrawn. For some experiments (Figure 4F) we attempted to resolve individual tapping events and the female was presented and immediately withdrawn. Different target flies were interleaved with female stimuli and the preparation was discarded if the response to the female stimulus degraded over time. Images were collected at 1-3hz (usually 1.7hz), and typical preparations could be imaged intermittently for an hour, allowing six sets of 3-6 bouts of regularly spaced tapping events and occasionally as many as 12 sets.

Antennaectomy or antennal nerve severing was performed on tethered animals prior to imaging using sharp forceps. The two surgeries produced similar results and are combined in Figure 5. Animals recovered for an hour in the chamber prior to head opening and imaging.

For P1 recordings, every male was tested with the virgin *D. melanogaster* female positive control, and most males were also tested with male *D. melanogaster*. Most males were also tested with one of three additional stimuli—virgin female *D. simulans*, virgin female *D. sechellia*, or mated female *D. melanogaster*—to make up separate experiments presented in the

figures. Mated female stimuli were mated no more than four hours prior to use in an experiment. For mAL and vAB3 recordings, all animals were stimulated with male and female *D. melanogaster* stimuli and a fraction were additionally tested with mated female stimuli. In Figure 5F, we only compare virgin and mated *D. melanogaster* female responses from the same experiment to minimize inter-experiment variability.

To stimulate with pure 7,11-HD, a fire polished glass electrode was dipped into 7,11-HD (10 mg/mL Cayman Chemicals) dissolved in ethanol and then allowed to evaporate to coat the glass.

To perfume virgin females with cVA, we applied 1ul of pure cVA (Cayman Chemicals) directly to a 1mm x 20mm strip of filter paper (Whatman Filter paper), placed inside of tubing (3mm OD, 1.5mm ID, 4cm in length). A virgin female was perfumed with cVA by allowing her to walk on the filter paper for 20 minutes and groom for 20 minutes. The quantity of cVA on females applied through this strategy was analyzed by thin layer chromatography and determined to be similar to the amount found on recently mated females (data not shown).

To represent responses graphically, we show heatmaps (F/F_0): the frame of peak fluorescence in response to a stimulus was divided by an average of the four frames immediately prior to stimulus presentation. To compare responses across animals, we calculated $\Delta F/F$ for each frame of calcium imaging time courses using the second to fifth frames as the baseline, producing traces as shown in Figure 1F. For vAB3, mAL, and P1 experiments, the quantified field of view corresponds to representative images in Figure 1F-G and 4A-B) and corresponds to the lateral protocerebral complex. For DC1 and DA1, regions similar to those boxed in Figure 6D were quantified. We selected the maximum from each stimulus bout e.g. as shown pooled in Figure 1H. For each experimental animal, we then averaged all peaks produced by a particular stimulus type (as in Figure 1I) and averaged these values across experiments to compare P1 responses to different stimuli as in Figure 1J, Figure S1C, and S5C. Normalizing each stimulus to its own positive control (virgin female) within an experiment or considering the peak of each stimulus bout (rather than the average for each animal) produced qualitatively similar results.

For 39 female stimulus bouts and 38 male stimulus bouts from 7 experiments, we aligned the movies of the target fly presentation with the GCaMP time series using the onset of 2p laser fluorescence through the head capsule. Frames in which tapping events occurred were scored manually. The trajectory of the stimulus towards the male across the 4mm frame of view was measured by making an ROI in ImageJ and using the Plot Z-Axis Profile function to generate a trace of the “inter-stimulus distance” in Figures 5 and 6. We used these trials to produce tap-triggered averages, aligned to the first tapping event. A similar analysis was conducted for odor presentation in Figure 6.

***In vitro* imaging, stimulation, and two-photon severing**

For *in vitro* imaging, the central nervous system (brain and ventral nerve chord) was dissected and the glial sheath softened with collagenase. The preparation was pinned onto Sylgard, and perfused with perfusion saline suffused with carbogen. Acetylcholine or ATP (to stimulate exogenously expressed P2X₂ channels) was locally applied using a fine glass electrode (~12 MOhm) driven by a micromanipulator and inserted into the target neuropil using visible light or fluorescent guidance. 10mM acetylcholine was applied using iontophoresis, with voltage

delivered and controlled through a Grass stimulator. 2mM ATP was applied using precisely controlled pressure pulses delivered by a custom made pressure-injector through the sidearm of a stimulating electrode. The amount of pressure or voltage required to deliver the stimulus was determined for each preparation but did not exceed 500ms at 8V (iontophoresis) or 200ms at 800mL/min (pressure injection), except for the voltage ramp experiment shown in Figure 4H,I (up to 20 V). Saline (negative) controls were performed for each type of experiment, as were ATP stimuli in animals lacking a Gal4 allele for P2X₂ experiments. Time courses were collected at 1-2hz. For quantitative comparisons of responses across experiments (Figure S2, 3F, 4I, 6H), we analyzed data from only a single Z plane. For P1 neurons, the quantified region of interest (ROI) corresponded to the boxed region of the lateral protocerebral complex shown in Figure 1D. For mAL and vAB3 neurons, ROIs were drawn on axon tracts as they left the SEZ, where they could be unambiguously identified.

For anatomic tracing of functionally responsive neurons (Figure 2, Figure S3A, C, Figure S5 and representative images in Figure 6H), we performed identical stimulations with acetylcholine or ATP while imaging at each Z plane (every 2.5 to 10 μ m) and then combined these to build a volume of the anterior ~100 μ m of the brain. The order of planes was randomized and positive control planes were interspersed to assess sample health.

Targeting the DA1 glomerulus with a stimulating electrode has been described previously and can be accomplished based on anatomic features of the glomerulus alone (Ruta et al., 2010). We initially developed our procedure to target vAB3 dendrites in the ventral nerve cord using lines in which ppk25 sensory neurons expressed Tomato and the Fruitless promoter was used to drive GCaMP expression. We inserted the electrode in the neuropil in regions fluorescing red, visualizing the electrode by coating it with Texas Red Dextran BSA. Stimulation in this region produced changes in GCaMP fluorescence in local vAB3 processes that propagated to the lateral protocerebral complex. After gaining confidence in our targeting in this genetic background, the site of vAB3 innervation could be reproducibly targeted using visible light alone or using expression of Tomato in Fru⁺ neurons as a guide. The appropriate site was the most ventral location in the first thoracic ganglion where neurites could be observed crossing the midline by light microscopy. The electrode was positioned slightly posterior and slightly lateral to the anterior midline of this tract (~five microns each). Acetylcholine appeared to diffuse only about 10 microns from the site of application, based on loss of vAB3 stimulation when the electrode was moved outside this radius. The local nature of the stimulation combined with the anatomically segregated sensory innervation of the ventral nerve cord (described in Figure 2) allowed restricted and reproducible stimulation. Stimulations too lateral, anterior, or dorsal produced no change in GCaMP fluorescence in Fru⁺ neurons or P1 neurons. Using pan-neuronal GCaMP, stimulations that failed to excite vAB3 usually excited Fruitless-negative ascending neurons similar in anatomy to Fruitless-negative neurons labeled by *AbdB*^{LDN}. These did not target the SEZ or the lateral protocerebral complex. We occasionally observed stimulation of what appeared to be local interneurons. To stimulate mAL dendrites directly, an electrode coated with Texas Red Dextran BSA was advanced to the appropriate location during two-photon imaging using expression of Tomato driven by Fru^{LexA} as a guide.

To sever mAL axon tracts using the two-photon laser, we used expression of Tomato driven by the Fruitless promoter as a guide. We focused 925 nm light on the axon tract at 8X optical zoom

and drew a precise ROI extending across the tract. We then increased laser power and performed bouts of 8-16 scans until a cavitation bubble was observed. On occasion, this method could not sever the tract, in which case the procedure was repeated using 850nm laser illumination. Stimulation experiments subsequent to severing were performed both immediately and after 10 minutes to ensure that changes due to severing were stable and not a consequence of acute tissue damage.

Anatomic tracing and image processing

To photolabel neurons, we located the neural structure of interest using 925 nm laser illumination, a wavelength that does not cause significant photoconversion, defined an ROI in PrairieView Software in a single Z-plane, and exposed the target area to 710 nm light (~10-30 mW at the back aperture of the objective) 100-300 times. After diffusion of the photoconverted fluorophores throughout the targeted neurons for 30-60 minutes, we imaged at 925 nm using 1µm steps. Images were sometimes collected using fluorescence unmixing to minimize crosstalk between channels.

Dye filling was accomplished through electroporation of highly concentrated (100 mg/mL) 3,000-Da Texas Red dextran (Invitrogen). Pulled glass electrodes were back-filled with the dextran dye and then connected to the output of a stimulator (Grass) and positioned in the target neuropil by visible light or fluorescent guidance. Voltage pulses (30-50V pulses, 5 ms, 2 Hz) were applied until the dye became visible in distal neural processes.

For clarity, we masked autofluorescence from the glial sheath and basal fluorescence from out-of-plane structures in photoactivation experiments and in the *Fru^{LexA}* expression pattern. To highlight P1 anatomy when using *R71G01-Gal4*, we segmented P1 neurons from the *R71G01-Gal4* expression pattern. Other neurons labeled by the R71G01 driver do not anatomically overlap with P1 neurons.

References

All references can be found in the main text.